***NMMP1*, a Matrix Metalloprotease in *Nicotiana benthamiana* Has a Role in Protection against Bacterial Infection**

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(Received on October 28, 2010; Accepted on November 19, 2010)

Plant matrix metalloproteases (MMPs) are a family of apoplastic metalloproteases closely related to human matrilysins. Up-regulation of *Nicotiana benthamiana* matrix metalloprotease 1 (*NMMP1*) expression by treatment with pathogens, ethephon and aging indicates that the gene is related to plant defense and the aging process through ethylene signaling. *NMMP1* expression was higher than in normal growth leaves following infection with an incompatible pathogen *Pseudomonas syringae* pv. tomato T1 or a compatible pathogen *P. syringae* pv. tabaci and in aged leaves. Transient overexpression of *NMMP1* in *N. benthamiana* leaves lowered the growth of *P. syringae* pv. tabaci. However, *NMMP1*-silenced leaves showed increased growth of *P. syringae* pv. tabaci. These data strongly suggest that *NMMP1* in *N. benthamiana* is a defense related gene, which is positively regulated by ethylene.

Keywords : Defense, matrix metalloprotease, overexpression, pathogen, virus-induced gene silencing (VIGS)

Plants are equipped with a large proteolytic machinery for housekeeping to remove non-functional proteins and to release amino acids for recycling (Baek and Choi, 2008; van der Hoorn, 2008). Plant proteases are an essential factor for regulating growth, development, stress responses, and pathogen defense (Coffeen and Wolpert, 2004; Dreher and Callis, 2007; van der Hoorn and Jones, 2004). Hundreds of genes encoding proteases have been identified. For example, at least 723 and 955 putative protease genes within the *Arabidopsis* and *Populus* genomes have been identified, respectively (Garcia-Lorenzo et al., 2006). Among the proteases, matrixins or matrix metallopeptidases are the proteases that degrade and remodel structural proteins within

the extracellular matrix (ECM) (Barett et al., 2004). As a complex structural entity surrounding and supporting cells, the ECM is composed of structural proteins, specialized proteins, and proteoglycans (Roberts, 1994).

Matrixins belong to the M10A family in Clan MA(M), according to the MEROPS database system (Rawlings et al., 2010). The M10A family of vertebrate matrixins includes a number of proteases, such as matrilysins, collagenases, gelatinases and stromalysins (Barrett et al., 2004). Matrixins, synthesized as prepro-enzymes, target ECM using a signal peptide (Nagase and Woessner, 1999). Activation of the pro-matrixins is achieved by *in vivo* proteases or by mercurial compounds and reactive oxygen *in vitro* (Nagase and Woessner, 1999). In animal systems, matrixins are involved in development, embryogenesis, organ morphogenesis, and wound healing, as well as in pathological processes such as cancer and arthritis (Barett et al., 2004).

Several matrixins have been isolated from plants, including soybean (Graham et al., 1991; Liu et al., 2001; McGeehan et al., 1992; Pak et al., 1997), *Arabidopsis* (Maidment et al., 1999; Golldack et al., 2002), cucumber (Delorme et al., 2000), alfalfa (Combiér et al., 2007), *Pinus taeda* (Ratnaparkhe et al., 2009), and *Nicotiana tabacum* (Schiermeyer, 2009). Matrixins have zinc at their catalytic center and are sensitive to EDTA (McGeehan et al., 1992). The first matrixin isolated from a plant was SMEP-1 (soybean metalloendoproteinase-1) from soybean leaves, which has both structural and biochemical similarities with vertebrate matrixins (Graham et al., 1991; McGeehan et al., 1992). *GmMMP2*, a second matrixin from soybean is up-regulated during infection by an oomycete pathogen *Phytophthora sojae* or the bacterial pathogen *P. syringae* pv. *glycinae* (Liu et al., 2001). Up-regulation of *GmMMP2* by pathogens might indicate involvement of the gene in defense; however, *GmMMP2* is not induced by the classical pathogen-induced signaling molecules, such as salicylic acid and jasmonic acid (Liu et al., 2001). Gain- or loss-of-function studies of *MtMMPL1*, a *Medicago truncatula* matrixin, demonstrated that the gene is a novel and specific marker for root and nodule infection by *Sinorhizobium meliloti* and regulates *Rhizobia* infections (Combiér et al.,

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2007). *NtMMP1*, the first tobacco matrixin gene, is constitutively expressed at a low level but up-regulated by bacterial pathogen infection (Schiermeyer et al., 2009). All of these studies strongly suggest that plant matrixins are involved in pathogen defense mechanisms.

Using a cDNA bioinformatics analysis of *Nicotiana benthamiana*, we isolated *NMMP1*, the first ECM metalloprotease 1 gene isolated from *N. benthamiana*. We investigated the role and regulation of *NMMP1* in response to compatible and incompatible pathogens by employing gain-of-function studies.

Materials and Methods

Growth conditions for *N. benthamiana* and bacterial pathogens. *N. benthamiana* seeds were germinated in commercially available horticultural crop soil and grown in a controlled environment room under a regime of 16/8 h light/dark at 23±2°C. Six-week-old *N. benthamiana* plants were used for the various pathogen or chemical treatments. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or from Duchefa (Haarlem, The Netherlands).

The inoculated bacterial pathogens included *Pseudomonas syringae* pv. tomato *T1* (*PsT1*), *P. syringae* pv. tabaci (*Psta*), and *P. syringae* pv. tabaci 11528 (*Psta* 11528). *Psta* 11528 is resistant to rifampicin at 100 mg/mL. Bacteria were grown at 28°C in King's B media containing (per liter): 20 g proteose peptone no. 3, 10 mL glycerol, 1.5 g K₂HPO₄, and 1.5 g MgSO₄. For bacterial infection, an overnight culture of bacterial cells was harvested at 4°C and 4,000 rpm for 10 min and re-suspended in sterile 10 mM MgCl₂ at a concentration of 0.01, 0.05, or 0.1 OD_{600nm}.

***NMMP1* isolation and RNA analysis.** Total RNA samples were extracted from the leaves of *N. benthamiana* using TRI reagent (MRC, Cincinnati, OH, USA), according to the manufacturer's instructions. The quality of the total RNA was determined on 1.2% agar gels, and the amounts were quantified by a spectrophotometer (ASP-3700, ACT-Gene, Piscataway, NJ, USA). Complementary DNA was constructed with an RT-PCR kit using oligo dT supplied by Elpis Biotech Inc. (Daejeon, Korea). *N. benthamiana* matrixin genes were searched by comparing the matrixin genes in *Arabidopsis* with the sequences of *N. tabacum* cDNAs stored at Gene index (<http://compbio.dfci.harvard.edu/tgi/plant.html>), and, finally, EST no. TC7253 was identified as a matrixin gene in tobacco. The *N. benthamiana* matrixin gene was isolated by PCR with the forward primer, 5'-CACAGACTTTAAAGCTCAAA TAC-3', and the reverse primer, 5'-GATTTATCTGTCTCAAAA-TAGAG-3'. The PCR was performed in a 20 mL volume

containing 100 nM of primers, 2 mL of cDNA as the template, and 2 units of *Taq* polymerase (Elpis Biotech) using a PCR program composed of 94°C for 2 min followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 40 s, and an extension at 72°C for 3 min. An approximate 1.4 kb PCR product was visualized by 1.2% agarose gel electrophoresis, and cloned into the pCR2.1-TOPO vector using a Topo TA cloning kit (Invitrogen, Carlsbad, CA, USA). The cloned gene was finally identified as a matrixin in *N. benthamiana* by sequencing at the NICEM sequencing facility (Seoul National University, Seoul, Korea). Sequence data have been deposited at the NCBI database under accession number GU441835.

Defense signaling molecule treatments. Defense signaling molecules and bacterial pathogens were inoculated into the leaf mesophyll tissues using an 1-mL needleless syringe. Chemicals were prepared at concentrations of 0.1 mM salicylic acid (SA), 0.1 mM methyl jasmonate (MJ), or 5 mM ethephone (ET). Compatible *Pssta* and *Psta* 11528 pathogens, and an incompatible *PsT1* pathogen (Almeida et al., 2009) were grown in King's B liquid media at 28°C and adjusted to 0.01, 0.05, or 0.1 OD_{600nm} in sterile 10 mM MgCl₂ solution. Leaves were harvested at 0 to 24 h after the treatments, and total RNA was extracted or frozen immediately in liquid nitrogen and stored at -80°C before being used for RNA extraction.

Transient overexpression of *NMMP1* by *Agrobacterium* infiltration. The cloned *NMMP1* gene was digested with *Xba*I-*Bam*HI and integrated into the 23001 plant gene expression vector. The 23001 plant gene expression vector was a kind gift from Dr. SY Kwon at KRIBB, which was made by inserting a 35S CaMV promoter and connecting a NOS terminator in the pCAMBIA2300 vector. The recombinant vector or 23001 vector insert was transferred to *A. tumefaciens* strain EHA105 using the freeze-thaw method described by An et al. (1987). *A. tumefaciens* transformed with *NMMP1* in the 23001 vector or only the 23001 vector was grown in YEP media enriched with rifampicin (50 µg/mL) and kanamycin (50 µg/mL) at 28°C. The overnight culture of *Agrobacterium* transformants was collected by centrifugation at 6,000 g for 10 min at 4°C, resuspended in 10 mM MgCl₂ at 0.5 A₆₀₀, acetosyringone was added at 150 µM/mL, and the culture was incubated at 22°C for 4 hrs. The incubated *Agrobacterium* transformants were inoculated into 6-week-old *N. benthamiana* leaves. Transient *NMMP1* over-expression was detected by extracting total RNAs, synthesizing cDNAs, and performing RT-PCRs with the primer set (forward: 5'-GCAAACTTCAACCTCAACACC-3' and reverse: 5'-TTTGCAAGCTCCACTCTTCG-3'). PCRs were conducted in a volume of 20 µL

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cacagactttaaaagctcaaatactactacaaattttttctatcttccatttttgaacaga
aaactcgaaatgaggattcctttatattatcgccattgttttagttcttagcttatctcca
      M R I P L F I A I V L V L S L S P
gcttcagctcatttcttcccaaatatcttcaatccctcctttattgaaacataaac
A S A H F F P N I S S I P P L L K P N N
actgcttgggatgctttccacaagttattgggtgtgcacgctggtcagaaagtcgatggc
T A W D A F H K L L G C H A G Q K V D G
ttagctaaaaatataaaatatttctataattttgggtatatttcttctttagtaacttt
L A K I K K Y F Y N F G Y I S S L S N F
acagatgactttgatgatgctcttgaatctgctctcaagacttaccagcaaaacttcaac
T D D F D D A L E S A L K T Y Q Q N F N
ctcaacaccaccgggtgtactcgacgcgcgacgattgagcatctcataagaccagatgt
L N T T G V L D A P T I E H L I R P R C
ggaaacgcgcatgtagttaatggcaccagttaccatgaactccggcaagccatcggcaggt
G N A D V V N G T S T M N S G K P S A G
tctcagaatattcacacagtgggccacttctcgtttttccgggaagaccacgggtggccg
S Q N I H T V A H F S F F P G R P R W P
gagagtaacagagatttgacatacgcgtttttaccgcagaacggctcgacggataacatt
E S N R D L T Y A F L P Q N G L T D N I
aaaagcgtgttctcacgtgcgttttgaccgggtgggtcggaagtaacccggtgaccttcacc
K S V F S R A F D R W S E V T P L T F T
gaaatagcttctgtttcaatcggcggatattaagatcgggtttttcgccggagaccacaac
E I A S F Q S A D I K I G F F A G D H N
gacggcgagccggttgatggtccgatggggacattagctcacgcgttttcgcccgcaggg
D G E P F D G P M G T L A H A F S P P G
gggcatttttcaacttggacggcgacgagaattgggtcatcgacggcgccgagattgttgaa
G H F H L D G D E N W V I D G A P I V E
gggaatttcttttcaatattatcggcggttagatcttgaatcggttgcggttcagaaatc
C N F F S I L S A V D L E S V A V H E I
gggcatttattgggtttgggccattcatccgtagaagattcgattatgttcccgagttta
G H L L G L G H S S V E D S I M F P S L
gcagcgggtaccggaagagtggagctcgcaaatgatgatattcagggagtcagggtgtta
A A G T R R V E L A N D D I Q G V Q V L
tacgggtctaaccacaaattttactgggccgaacacagttttaactccgacgcagcaaaat
Y G S N P N F T G P N T V L T P T H E N
gacacaaatggagccctgaaatttgggttcattatgggttcacgtggttcgttcattcttc
D T N G A L K F G S L W V H V V V A F F
ttatcatttctcggtttaatttaaaaagttatacttctgtattatttgattcattttta
L S F L R L I -
aaaatttagaatatcatatcgctcattacgttgtcttttaattaagattgtttccttgatat
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taatttcattatgcttgtaaatggttatatttatgtatagttttaactattctcttttga
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ttgaataaaaataaaaaatcgactctattttgagacagataaatca
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Fig. 1. The nucleotide and amino acid sequence of the *Nicotiana benthamiana* matrix metalloprotease 1 (*NMMP1*) gene. The underlined and dotted underlined region were used for building the *NMMP1-M* or *NMMP1-3'UTR* gene silencing constructs, respectively.

containing 100 nM of primers, 1 μ L of cDNA as the template, and 2 units of *Taq* polymerase (Elpis Biotech, Korea). The PCR program was composed of 94°C for 3 min followed by 28 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 40 s, and extension at 72°C for 2 min. The PCR product was visualized on 1.2% agarose gel electrophoresis.

Virus-induced gene silencing (VIGS) of *NMMP1* in *N. benthamiana*. A stock solution of *A. tumefaciens* strain GV2260 was prepared for VIGS as described by Liu et al. (2002). Two VIGS constructs were prepared, such as *NMMP1-M* (targeting the *NMMP1* Zn-metalloprotease conserved region) and *NMMP1-3'UTR* (targeting the *NMMP1* 3'-untranslated region). PCRs were performed with specific primers to make the two constructs. The primer sets used to amplify *NMMP1-M* (the underlined region in Fig. 1) were the forward primer 5'-CGGAATTCGGAAGTAACCCGTTGAC-3' and the reverse primer 5'-TCCGCTCGAGATTGCGAGCTCCACTC-3'. The *NMMP1-3'UTR* fragment (the dotted underlined region in Fig. 1) was amplified using

the forward primer 5'-CGGAATTCAAAAGTTATACTTCTGTATTA-3' and the reverse primer 5'-TCCGCTCGAGAAAAGTTATACTTCTG-3'. The amplified fragments were digested with *EcoRI* and *XhoI* and ligated into TRV2 plasmids. *A. tumefaciens* was transformed with TRV2 plasmids containing the genes of green fluorescent protein (*GFP*), pepper phytoene synthase (*CaPDS*) (Chung et al., 2004), *NMMP1-M*, or *NMMP1-3'UTR*, respectively. Single colonies of *Agrobacterium* transformed with these gene fragments into the TRV2 vector were grown overnight in 20 mL of YEP media containing rifampicin (50 μ g/mL) and kanamycin (50 μ g/mL). The transformed *Agrobacterium* was harvested by centrifugation at 4,000 rpm for 15 min at 4°C, re-suspended to 0.4 A_{600nm} in 10 mM $MgCl_2$ with 150 μ M acetosyringone, and incubated at 22°C with shaking for 4 h. *A. tumefaciens* transformed with TRV1 was adjusted to a concentration of 0.08 A_{600nm} in 10 mM $MgCl_2$ with 150 μ M acetosyringone and incubated at 22°C with shaking for 4 h. *A. tumefaciens* transformed with TRV1 and *A. tumefaciens* transformed with TRV2:*GFP*, TRV2:*CaPDS*, TRV2:*NMMP1-M*, or TRV2:*NMMP1-3'UTR* were mixed at

a 1:1 ratio and then inoculated into the cotyledons of two-week old *N. benthamiana* seedlings using an 1-mL needleless syringe. At least 12 seedlings were used for silencing *NMMP1*. After 20 days of VIGS treatment, the silenced *N. benthamiana* were inoculated with *Psta*, *Psta* 11528, or *PstT1* at concentrations of 0.01, 0.05, and 0.1 OD_{600nm}. All experiments were repeated at least twice.

Effect of *NMMP1* over-expression or silencing on pathogen attack. The 6-week old leaves of *N. benthamiana* over-expressing or silencing *NMMP1* were inoculated with *Psta* 11528 or *PstT1*. The index of *Psta* 11528 or *PstT1* infection was determined based on the bacterial colony-forming units (CFUs) or by visual examination of necrotic regions, respectively. The CFUs were counted on 2 days of bacterial growth on the leaves. For the bacterial growth count, 0.9-cm leaf discs were ground with 3 mL of 10 mM MgCl₂ solution, and 10 µL of diluted cells were layered onto King's B media with 100 µg/mL rifampicin. The CFU pathogen growth data were analyzed by Tukey's two-way analysis of variance using SAS version 6.12 (SAS Inc., Cary, NC, USA). The experiments were repeated at least three times.

Results

***NMMP1* sequence analysis in *N. benthamiana*.** The isolated *NMMP1* cDNA encodes a full-length protein with an open reading frame of 364 amino acid residues (Fig. 1), and the amino acid sequence was deposited in the NCBI database (accession no. ADD21635). *NMMP1* shares 95%, 82.9%, and 82.2%, amino acid identity with the matrixin genes from tobacco, tomato, and pepper, respectively (Fig. 2). Multiple sequence alignments of the *NMMP1* protein revealed that *NMMP1* has a highly conserved Zn-containing catalytic protease domain for matrix metalloprotease. *NMMP1* is predicted to have a cell surface glycosylphosphatidylinositol (GPI)-anchor site at amino acid 340 by the big-PI Predictor (Eisenhaber et al., 1999), suggesting attachment of the protein on the plasma membrane by the GPI-anchor.

Expression of *NMMP1* in response defense signaling molecules. *NMMP1* expression was detected in *N. benthamiana* treated with pathogens and various defense signaling molecules to understand the role of *NMMP1*. *NMMP1* expression in leaves treated with *Psta* and *PstT1* was determined for 24 h (Fig. 3). When leaves of *N. benthamiana*

Fig. 2. Amino acid sequence alignment of the matrixin proteins of *Nicotiana benthamiana*, tobacco, tomato, and pepper using ClustalW. Translated sequences of *N. benthamiana* matrix metalloprotease 1 (NMMP1; NCBI ADD21635), tobacco matrixin gene (accession no. CAA77093), pepper matrixin EST contig (canc16709), and tomato matrixin gene (NCBI accession no. AK322919) were aligned to compare the sequences.

Fig. 3. Analysis of the *Nicotiana benthamiana* matrix metalloprotease 1 (*NMMP1*) expression patterns in response to biotic stresses and defense signal molecules. *NMMP1* expression in *N. benthamiana* was determined by semi-quantitative RT-PCR. *N. benthamiana* leaves were inoculated with 10 mM MES, 10 mM MgCl_2 (control), a compatible pathogen *Pseudomonas syringae* pv. tabaci (*Psta*) at 0.1 $\text{OD}_{600\text{nm}}$, an incompatible pathogen *P. syringae* pv. tomato T1 (*PsT1*) at 0.1 $\text{OD}_{600\text{nm}}$, or defense signaling molecules including 1 mM salicylic acid (SA), 0.1 mM methyl jasmonate (MJ), or 5 mM ethephon (ET). RNA was isolated from the treated leaves, and semi-quantitative RT-PCR was performed using the RNA as the cDNA templates.

were treated with a compatible pathogen *Psta* and an incompatible pathogen *PsT1*, the *NMMP1* expression levels increased significantly after 3 h of treatment. The *NMMP1* expression levels in leaves treated with *Psta* decreased after 6 h; however, the expression levels increased again after 12 h of treatment. The *NMMP1* expression levels in leaves treated with *PsT1* increased significantly, but decreased after 24 h, when the *N. benthamiana* cells were dying due to a hypersensitive response (HR).

To test which signal molecules regulate *NMMP1* expression, *N. benthamiana* leaves were inoculated with defense signaling molecules, such as SA, MJ, and ET for ethylene (Fig. 3). Further characterization of *NMMP1* gene expression after 24 h of defense signal molecule inoculation revealed that only ET highly induced *NMMP1* up-regulation after 3 h of treatment.

Role of *NMMP1* in *N. benthamiana* pathogen defense.

The role of the *NMMP1* gene in defense was investigated by employing transient *NMMP1* overexpression or silencing in *N. benthamiana* and in plants infected with *Psta* 11528 or *PsT1*. Transient *NMMP1* over-expression in *N. benthamiana* was verified by semi-quantitative RT-PCR (Fig. 4a). After one day inoculation of *Psta* 11528, the control leaves had a 1.59 times greater number of CFUs than the leaves over-expressing *NMMP1* (Fig. 4b).

Fig. 4. Effect of *Nicotiana benthamiana* matrix metalloprotease 1 (*NMMP1*) overexpression on the growth of a compatible pathogen *Pseudomonas syringae* pv. tabaci 11528 (*Psta* 11528). (a) Semi-quantitative RT-PCR detecting *NMMP1* expression levels in a leaf of wild-type *N. benthamiana* (lane 2), 23001 vector only-expressed *N. benthamiana* (lane 3), and *NMMP1* over-expressed *N. benthamiana* (lane 4). Size markers (one kb plus markers) were loaded in lane 1. (b) Growth of *Psta* 11528 inoculated at 0.01 $\text{OD}_{600\text{nm}}$ in 23001 vector-expressed or *NMMP1* over-expressed leaves.

NMMP1 over-expressed leaves seemed to show faster HR symptoms in response to infiltration of the *PstI* incompatible pathogen than control leaves inoculated with only the 23001 vector itself (data not shown); however, it was difficult to determine the exact time for induction of the HR symptoms.

The role of *NMMP1* in the pathogen defense response was further investigated by silencing *NMMP1* using the VIGS technique. Two VIGS vectors were constructed; *NMMP1-M* containing the matrixin catalytic region and *NMMP1-3'UTR* containing the 3' UTR. *NMMP1*-silenced *N. benthamiana* showed no significant morphological changes compared to wild type or GFP-silenced *N. benthamiana*. Semi-quantitative RT-PCR data showed that the *NMMP1*-silenced plants had reduced *NMMP1* expression compared to the *GFP*-silenced control plants (Fig. 5a). When the growth of *Psta* 11528 was determined by counting CFUs from the pathogen infiltrated leaves, *NMMP1*-silenced leaves infected with *Psta* 11528 had significantly higher numbers of CFUs than the control leaves after 2 days of infection (Fig. 5b). Visual inspection did not show any slower induction of the HR symptoms in *NMMP1*-

Fig. 5. Effect of *Nicotiana benthamiana* matrix metalloprotease 1 (NMMP1) silencing on the growth of a compatible pathogen *Pseudomonas syringae* pv. *tabaci* 11528 (*Psta* 11528). Two virus-induced gene silencing vectors NMMP1-M or NMMP1-3UTR were constructed to silence the catalytic middle region or the 3' untranslated region of NMMP1, respectively. Leaves silenced with green fluorescent protein (control), NMMP1-M, or NMMP1-3UTR were inoculated with *Psta* 11528, and colony forming units (CFUs) were counted over 2 days. (a) Semi-quantitative RT-PCR analysis showing NMMP1 expression levels in *N. benthamiana* leaves under normal growth conditions (lane 1: control, lane 2: NMMP1-M silenced, lane 3: NMMP1-3UTR silenced), after 1 day of *Psta* 11528 inoculation (lane 4: control, lane 5: NMMP1-M silenced, lane 6: NMMP1-3UTR silenced), and after 2 days of *Psta* 11528 inoculation (lane 7: control, lane 8: NMMP1-M silenced, lane 9: NMMP1-3UTR silenced). M represents the loading of one kb plus marker. (b) Growth of *Psta* 11528 inoculated into the leaves of GFP-silenced or NMMP1-silenced *N. benthamiana*. CFUs of *Psta* 11528 inoculated at 0.01 OD_{600nm} were counted after 2 days. The CFU pathogen growth was analyzed by Tukey's two-way analysis of variance using SAS. Values followed by different letters are significantly different ($p < 0.05$).

silenced plants than in the GFP-silenced control plants (data not shown).

Discussion

The ECM is a complex structural entity surrounding and supporting cells and is the boundary for plant pathogens and plant cell interactions. The roles of matrixins as determinant proteases of ECM structure have been studied for growth, development, and pathogenesis (Barett et al., 2004). However, the roles of plant MMPs in pathogen

defense are unclear, although there has been speculation that MMPs are involved in plant growth, development, and defense responses (Golldack et al., 2002). We isolated the NMMP1 gene, which is the first time an ECM metalloprotease gene has been isolated in *N. benthamiana*. The NMMP1 gene has an open reading frame of 1,095 nucleotides; therefore, it encodes a 364 amino acid protein. As a protein predicted to be connected to the plasma membrane by a GPI anchor, the C-terminus of the mature NMMP1 protein is attached to glycosylphosphatidylinositol by posttranslational modification. The two fatty acids within the hydrophobic phosphatidyl-inositol group anchor the NMMP1 protein to the plasma membrane, so the NMMP1 protein remains attached to the exterior leaflet of the plasma membrane.

NMMP1 was constitutively expressed at very low levels; however, it was rapidly up-regulated by compatible and incompatible pathogen infections. Using the transient over-expression technique, we increased NMMP1 gene expression levels by inoculating *Agrobacterium* with NMMP1 into the leaves of *N. benthamiana*. Successful NMMP1 over-expression decreased the growth of the *Psta* 11528 (Fig. 4c). In contrast to the over-expression experiments, silencing NMMP1 in *N. benthamiana* using the VIGS technique resulted in significantly faster growth of *Psta* 11528 (Fig. 5b). We further investigated the defense signal molecules that regulate NMMP1 expression and found the involvement of ET (Fig. 3). Other defense molecules, including SA and MJ did not change NMMP1 expression levels, which coincided with previous reports of matrixin expression in other plant species (Liu et al., 2001; Schiermeyer et al., 2009). Only the ET treatment, the precursor of ethylene signal molecule, induced high expression of NMMP1. These data strongly suggest that the newly isolated NMMP1 gene is regulated by ethylene signaling, and that there is active involvement of the gene product in defense to reduce the spread of bacterial pathogens. The higher production of ethylene by pathogen attack induces the over-expression of NMMP1, which might activate the defense systems in ECM.

A huge accumulation of evidence has implicated the involvement of proteases in pathogen defense. The NMMP1 in *N. benthamiana* is constitutively expressed in small amounts and secreted to ECM but attached to the plasma membrane. When *N. benthamiana* ages or is severely threatened by bacterial pathogens, overproduction of ethylene gas can trigger NMMP1 expression. However, the involvement of NMMP1 in aging was not evident in the NMMP1-silenced *N. benthamiana*, suggesting that NMMP1 over-expression during senescence might not be directly correlated with aging. Identification of the NMMP1 target proteins in host plants for pathogen defense will be chal-

enging to understand the function of NMMP1 in pathogen defense. Many GPI-anchored proteins are preferentially located in lipid rafts and released from the membrane by phospholipase C action, therefore, identifying the location of NMMP1 on the plasma membrane and the involvement of phospholipase C in NMMP1 release will be interesting work for the future.

Acknowledgements

This work was financially supported by the Yeungnam University Research Grants in 208-C-000-162 to K.-H. Baek.

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